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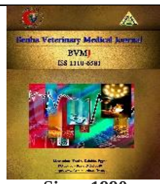

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Original Paper

Ameliorative effects of induced pluripotent stem cells on experimentally induced ovarian ablation in female rats

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ABSTRACT

The current study designed to evaluate the role of pluripotent stem cells (iPSc) on experimentally-induced ovarian ablation (OA) by evaluating hematological parameters, FSH and E2 changes, molecular and histopathological examination. 50 female rats were divided into five groups with 10 in each group. Group (1): control group. Group (2): rats injected with Doxorubicin (DOX) (3 mg/kg) and Cyclophosphamide (CYP) (50mg/kg) dissolved in sterile physiological saline injected I/P once a week for 5 weeks. Group (3): 10 rats undergo chemoablation then injected with 5 IU of pregnant mare serum gonadotropin (PMSG) single s/c injection. Group (4): 10 rats undergo chemoablation, and then iPSc injected i/v single injection for 2 months. Group (5): 10 rats undergo chemoablation then iPSc injected i/v combined with single s/c injection of 5 IU PMSG. Results revealed that rats injected with Dox and CYP showed a significant decrease ($p \leq 0.05$) in RBCs, Hb and PCV, WBCs, lymphocytes, neutrophil, eosinophil, E2 level and Oct 4 while there was significant increase ($p \leq 0.05$) in MCV, MCH, monocytes and FSH level compared with control (-ve) group. While, rats treated with PMSG or iPSc or PMSG and iPSc revealed significant increase ($p \leq 0.05$) in RBCs count as well as MCHC, E2 level and Oct 4.In addition, there was a significant increase ($p \leq 0.05$) of neutrophil in iPSc or PMSG and iPSc groups together with a significant decrease ($p \leq 0.05$) in monocytes compared to OA group and control (-ve) group.

1. INTRODUCTION

The ovaries have a fundamental role in reproduction and the production of hormones and egg cells. Also, granulosa cells and theca cells found in the ovary secrete multiple hormones, including estrogen and progesterone (Tetkova et al., 2019). Functional ovaries are necessary to maintain fertility as well as hormonal balance during the reproductive years (Fraison et al., 2019). Premature ovarian failure (POF) is a disease characterized by estrogen deficiency, reduced follicles and elevated gonadotropin (Robles et al., 2013). It may be caused by numerous factors, as autoimmune reactions, chemotherapy, radiotherapy, surgery, and endocrine dysfunction (Goswami and Conway, 2007).

The ovary is the most sensitive organ to chemotherapy drugs (Oktem and Oktay, 2007). Doxorubicin and Cyclophosphamide induce double-stranded DNA breaks in primordial follicles, which trigger the apoptotic process and death of follicles (Titus and Oktay, 2014). DOX and CYP affects ovarian functions by rapid depletion of the 0ocyte reserve which was mediated by prevention of cell division with disappearance of resting primordial follicles and growing follicles (Salama et al., 2013).

Stem cells have the ability of proliferation, self-maintenance, and differentiation into functional progeny with flexibility or plasticity (Vats et al., 2005).

Pluripotent stem cells (iPSc) "embryonic stem cell-like" cells were derived from the reprogramming of adult somatic cells by introduction of specific pluripotent associated genes (Omole and Fakoya, 2018). It provided a novel strategy for preserving or recovering damaged ovarian function of women who receive chemotherapy. iPSCs were obtained by the over-expression of four transcription factors: Oct4, Sox_2, Klf_4 and c-Myc by retrovirus-mediated transduction of fibroblasts (Takahashi and Yamanaka, 2006). Stem cells migrate into injured ovarian tissue and differentiate into ovarian tissue-like cells, particularly into granulosa cells, which have a key role in regulating reproductive ovarian physiology (Jiang et al., 2016).

The present study designed to evaluate the effects of iPSc on experimentally-induced ovarian ablation (OA) by evaluating of hematological parameters, FSH, E2 level changes, molecular and histopathological examination.

2. MATERIAL AND METHODS

2.1. Animals

In this study, 50 female rats were divided into five groups (10 rat /cage) in room temperature, for a week before starting the experiment.

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2.2. Methods

2.2.1. Experimental design

In this experiment, 50 adult rats were divided into five groups as following:

Group 1: 10 rats control negative.

Group 2: 10 rats injected I/P with DOX (5 mg/kg) according to Jagetia and Lalnuntluangi (2016) and CYP (50mg/kg) dissolved in sterile physiological saline once a week for 5 weeks according to Alison and Brian (2010).

Group 3: 10 rats undergo chemoablation, then injected with 5 IU of PMSG single s/c injection .

Group 4: 10 rats undergo chemoablation, then iPSc injected single injection for 2 months.

Group 5: 10 rats undergo chemoablation then iPSc injected i/v combined with single s/c injection of 5 IU PMSG.

2.2.2. Sampling

*2.2.2.*1. Whole blood: About 2 ml blood was received on vacuum tubes containing 3.6 mg EDTA used for complete blood picture.

*2.2.2.*2. Serum samples: blood was collected in plain clean well dried centrifuge tubes for separation of serum to be used in estimation of biochemical parameters.

Blood samples were allowed to clot, then obtained by centrifugation at 3000 round per 15 minutes then kept in deep freezer (-18ºc) till examination.

*2.2.2.*3. Tissue specimens: Ovary specimens collected after sacrificing then preserved in neutral buffer formalin solution (10%) for histopathological and frozen ovarian sections for PCR examinations .

2.2.3. Clinicopathological examinations

2.2.3.1. Haematological studies

The haematological studies including erythrogram, leukogram counts were determined according to Bourdon et al., (1999.)

- The erythrogram including hemoglobin concentration (Hb), Packed cell volume (PCV %), RBCs count and red blood indices.
- The leukogram including total leukocytic count and differential leucocytic counts were measured on hematology analyzer Exigo–VET, (Stockholm, Sweden).

2.2.4. Assessment of biochemical parameters:

FSH level was determined according to Rat FSH ELISA Kit (manufactured by Elabscience Company. Cat No: E-EL-R0391. E2 was measured by using Rat E2 ELISA Kit (manufactured by Elabscience Company. Cat No: E-EL-0152.

2.2.5. Determination Oct4

2.2.5.1. Total RNA extraction and reverse transcription:

Total RNA was extracted from tissue samples using a RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. For cDNA synthesis, extracted RNA samples were quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA) and (1 µg) reverse transcribed with a T100 Thermal Cycler (Bio-Rad, USA) using a QuantiTect Reverse Transcription Kit (Qiagen, Germany), following the manufacturer (Sriraman et al., 2015).

2.2.5.2. Quantitative real-time PCR

Real-time PCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen, Germany) on a Step One Plus Real-Time PCR System (Life Technologies, USA) according to Schimmer at al., (1998).

The sequences of the specific primers used were as follows:

Oct-4A:	
5′-CCATGTCCGCCCGCATACGA-3′ (forward)	
5′- GGGCTTTCATGTCCTGGGACTCCT-3′ (reverse)	
GAPDH:	
5′-ACCACAGTCCATGCCATCAC-3′ (forward)	
5′-TCCACCACCCTGTTGCTGTA-3′ (reverse)	

(Schimmer et al., 1998).

2.2.6. Statistical analysis

All the data were recorded and analyzed using IBM SPSS Statistics software for Windows, Version 22 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) with Post Hoc LSD test applied to compare differences among the groups. The differences considered significant (sig.) at $P < 0.05$.

3.1. Haematological changes.

3.1.1. Erythrogram results:

Erythrogram parameters were summarized in table (1). Rats injected with DOX and CYP showed significant decrease in RBCs, Hb and PCV values, while there was a significant increase in MCV and MCH compared with corresponding control group. While after treatment with iPSc with or without PMSG, there were significant increase in RBCs and MCHC with non-significant changes in Hb and PCV, while there was a significant decrease in MCV and MCH compared with OA group.

3.1.2. Leukogram changes

The leukogram data are illustrated in table (2). There were a significant decrease in total WBCs, lymphocytes, neutrophil, eosinophil while there were a significant increase monocyte in DOX and CYP injected rats compared with control (-ve) group. Rats treated with PMSG or iPSc or PMSG and iPSc showed non-significant changes in WBCs, lymphocytes, eosinophil counts with a significant increase of neutrophil in iPSc or PMSG and iPSc groups and a significant decrease in monocytes compared with control (OA)group and control (-ve) group .

3.2. Biochemical results

Table (3) illustrated FSH and E2 levels. Results showed that Dox and CYP injected rats showed significant decrease in E2 level compared with control (-ve) group, while rats treated with PMSG or iPSc or PMSG and IPSc revealed significant increase in E2 level compared with OA group. Also, There are non-significant changes between group injected with iPSc and PMSG and their control (-ve) group. There were significant increase in FSH level in OA group when compared with control (-ve) group.While, there was significant decrease in FSH level in group treated with PMSG or iPSc or iPSc compared with their OA group.

3.3. Results of Oct 4 gene expression

Table (3) illustrated Oct 4 gene expression. DOX and CYP injected rats showed a significant decrease in Oct4 when compared with control (-ve) group . While after injection of iPSc or PMSG or iPSc and PMSG, there were a significant increase in Oct4 compared with OA control group. Also, there were a significant increase between group treated with iPSc only or iPSc and PMSG compared with control (-ve) group.

3.4. Histopathological results

The ovary of normal group showing follicles in different developmental stages: primary, secondary, and tertiary follicles. While, ovary of OA group showing reduction of primordial and primary follicles. Ovary of rats of hormonal treated group PMSG treated group revealing development of follicles in normal sequence, but in rudeminted number Also, ovary of rats of iPSc treated group revealing ovary with normal architecture with high number of anular follicles. Ovary of rats of hormonal and iPSc treated group showing high incidence of follicles in advanced stages of developments.

Table 1 RBCs, Hb, PCV and Red blood indices after treatment in different groups.

Groups	RBCS (x10^{6}/µl)	Hb (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (%)
Group (1)	8.31±0.20^{c}	14.87±0.43^{c}	42.57±1.49^{b}	51.23±0.96^{a,b}	17.87±0.21^{a}	34.9±0.24^{a}
Group (2)	5.78±0.57^{a}	12.13±1.17^{a}	34.8±2.75^{a}	60.4±1.25^{c}	21±0.05^{c}	34.7±0.63^{a}
Group (3)	7.02±0.11^{b}	13.47±0.12^{a,b}	37.6±0.06^{a,b}	53.5±0.91^{b}	19.2±0.49^{b}	35.87±0.32^{b}
Group (4)	7.54±0.13^{b,c}	13.37±0.52^{a,b}	36.77±1.7^{a}	48.7±1.83^{a}	17.7±0.55^{a}	36.4±0.35^{b}
Group (5)	7.72±0.32^{b,c}	13.73±0.39^{a,b}	38.5±0.98^{a,b}	49.97±1.18^{a,b}	17.8±0.34^{a}	35.7±0.18^{a,b}

Data are presented as Mean ± SE. SE = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

Table (2): Total leucocytes, differential leucocytic counts in different groups.

Groups	WBCs (x10^{3}/µl)	Lymphocytes (x10^{3}/µl)	Neutrophil (x10^{3}/µl)	Monocytes (x10^{3}/µl)	Eosinophil (x103/µl)
Group (1)	11.3±0.67^{b}	7.97±0.61^{b}	2.43±0.22^{a}	0.39±0.17^{a}	0.51±0.51^{b}
Group (2)	7.77±1.18^{a}	4.53±0.67^{a}	1.47±0.23^{b}	0.70±0.09^{b}	0.12±0.7^{a}
Group (3)	8.83±0.87^{a,b}	6.28±0.22^{a,b}	1.97±0.44^{b}	0.34±0.21^{a,b}	0.18±0.7^{a}
Group (4)	9.33±0.09^{a,b}	6.53±0.37^{a,b}	2.40±0.39^{a,c}	0.16±0.03^{a}	0.25±0.7^{a}
Group (5)	9.32±1.13^{a,b}	6.33±0.44^{a,b}	2.31±0.46^{d}	0.36±0.15^{a,b}	0.23±0.09^{a}

Data are presented as Mean ± SE. SE = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

Table (3): E2 and FSH Oct4 levels after treatment in different groups.

Groups	E2 (pg/ml)	FSH (mIU /ml)	Oct 4
Group (1)	51±3.9^{b,c}	40±3.9^{a}	1.044±0.06^{b}
Group (2)	13.65±3.4^{a}	106±12.9^{c}	0.81±0.01^{a}
Group (3)	42.6±3.4^{b}	67±2.95^{b}	1.33±0.05^{c}
Group (4)	44.4±2.8^{b}	66±2.06^{b}	2.11±0.11^{d}
Group (5)	63.98±4.46^{c}	66.4±4.62^{b}	2.09±0.05^{c}

Data are presented as Mean ± SE. SE = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

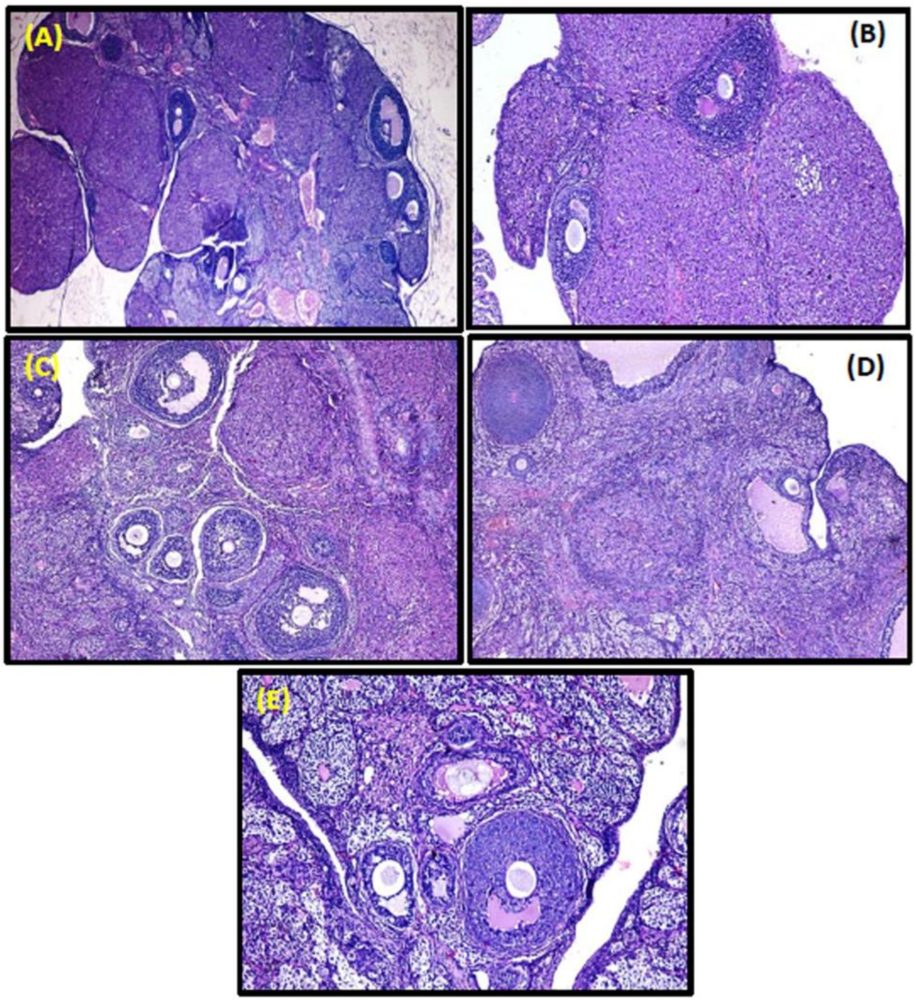

Figure 1 Revealing folliculogenesis in different groups: (A) Ovary of normal group showing follicles in different developmental stages: primary, secondary and tertiary follicles. (B) Ovary of OA group revealing reduction of primordial and primary follicles. (C) Ovary of hormonal treated group showing normal architecture harboring high incidence of anular follicles. (D) Ovary of iPSc treated group showing initiation of primordial and primary follicles in rudimentary number. (E) Ovary of rats of hormonal- iPSc treated group showing high incidence of follicles in advanced stages of developments. Primordial (pd), Primary (p), secondary (s), Tertiary (t), mature Graafian (m) follicles (H&E x200).

4. DISCUSSION

POF is the partial or total loss of reproductive and hormonal function of the ovaries because of follicular dysfunction or early loss of eggs (Martin et al., 2017). IPSc named as artificial stem cells produced from somatic cells through expression of defined pluripotency-associated factors (Takahashi et al., 2007).

According to the erythrogram results, Dox and CYP injected rats showed decrease RBCs, Hb and PCV. These results agreed with Kandemirmt al. (2005), Liu et al. (2009) and Shrivastava et al. (2017). They reported that low blood counts are observed as a side effect of chemotherapies due to decline in the number and the life span of RBC. The decrease in PCV might be the consequence of erythropoiesis failure, destruction of mature cells and increased plasma volume. While, reduction in Hb due to blockage of the incorporation of iron into hemoglobin due to disturbance in the bio-generation structure of hemoglobin molecule, or oxidation of hemoglobin iron causing loss of the biological structure and activity of hemoglobin molecule. While after treatment with PMSG or iPSc or iPSc and PMSG, data demonstrating increase in RBCs as well as MCHC with non-significant changes in Hb and PCV. iPSc support hematopoiesis by creating an optimal microenvironment by providing cytokines to stimulate and enhance proliferation of the hematopoietic elements (Cheng et al., 1998).

According to the results, there was decrease in total WBCs, lymphocytes, neutrophil, eosinophil while there was increase monocyte in DOX and CYP injected rats compared with control (-ve) group. These results agree with Aziz and Habeeb (2019). They indicated decrease in WBCs of patients receiving anticancer treatment due to defect in bone marrow production of these cells. while, rats treated with PMSG or iPSc or PMSG and iPSc showed no changes in WBCs, lymphocytes, eosinophil counts with a significant increase of neutrophil IPSc or PMSG and IPSc groups and a significant decrease in monocytes compared with control (OA)group and control (-ve) group. These data partially disagree with Aggarwal and Pittenger (2005), they stated that stem cells have the ability to modify and influence almost all the cells of the innate and adaptive immune systems to interfere with cellular proliferation, differentiation, maturation, and functions.

According to the result, Dox and CYP injected rats showed decrease in E2 level. These results agree with Petrillo et al. (2011) and Jiang et al. (2013). E2 is produced especially within the follicles of the ovaries. Chemotherapy lead to cytotoxicity to dividing cells which leads to a loss of fertility and reproductive endocrine functions. Chemotherapeutic drugs caused a decrease in healthy primordial follicles and small primary follicles in both mouse and rat ovaries. These biochemical results confirmed to the histopathological changes of rat administered Dox and CYP, the ovary showing marked reduction of primordial and primary follicles associated with persistence of anular follicles. These results agree with McLaren and Bates, (2012) and Amira (2017). Data showed increase in E2 level after treatment with iPSc. This supported by Anchan et al. (2015), Jiang et al. (2016) and Lipskind et al. (2017). They reported that Stem cells migrate into injured ovarian tissue and differentiate into ovarian tissue-like cells, particularly granulosa cells..

Our data showing, there were increase in FSH level in rats injected with DOX and CYP. These data agree with Zheng et al. (2019) and Jiang et al. (2020). They reported that chemotherapeutic drugs led to DNA damage and pyroptosis of granulosa cells, so resulted in fall in E2. The fall in E2 elevated FSH via negative feedback mechanisms. While data showed decrease in FSH level in rats injected with iPSc. This supported by Anchan et al. (2015), Lipskind et al. (2017). IPSc migrate into injured ovarian tissue and differentiate into ovarian tissue-like cells, particularly into granulosa cells, which are an essential component of the ovarian micro-environment and have a key role in regulating reproductive ovarian physiology.

Concerning to OCT4, results showed that DOX and CYP injected rats showed decrease in OCT4 gene expression. This data agree with Titus and Oktay, (2014). They revealed that reproductive functions deteriorate by rapid depletion of the oocyte reserve mediated by apoptotic cell death and ovarian atrophy with disappearance of resting primordial follicles and also growing follicles. It reported that OCT_4 detected in oocyte and granulosa cell of growing follicles which plays important roles in oocyte growth and meiosis. While data showed increase in OCT4 in rats injected of iPSc or PMSG or IPSc and PMSG when compared with OA group. This supported by Zou et al. (2009) and Shi and Jin, (2010). They reported that Oct4 is a regulator of pluripotency expressed in pluripotent stem cells, can induce pluripotency in somatic cells upon transfection.

5. CONCULOSIONS

From the present study we could conclude that there were improvements in hematological parameters, ovarian functions (FSH and E2) and OCT4 as well as histopathological changes after treatment with iPSc or iPSc and PMSG compared with DOX and CYP with superiority of iPSc and PMSG together.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to disclose.

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